THE BACTERIAL INTEGRITY OF COLLODION SACS.*

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THE use of collodion sacs and parchment membranes for the study of the longevity of B. typhosus in natural waters and sewage was introduced by Jordan, Russell, and Zeit[1] in 1904 in their work on the Chicago Drainage Canal case. The particular advantages of this technic over the methods employed up to that time are that the typhoid bacilli are confined within comparatively narrow limits and can thus be recovered more easily than otherwise, and that at the same time the organisms thus confined are exposed to conditions as nearly as possible like those found in nature, since there is quite a free interchange of diffusible substances through the permeable walls of the sacs. In 1905–6 Russell and Fuller[2] used similar containers in a more extended study of the vitality of the typhoid bacillus in surface waters and sewage.

Recently the entire reliability of the results obtained by the use of collodion and parchment containers has been questioned on the ground that it is not possible to prepare either collodion or parchment sacs through which the typhoid organism will not pass in a few hours, and that the figures reported in the above-mentioned experiment are unreliable because they do not take into account the escape of a certain number of the typhoid bacilli through the walls of the sacs during their exposure in water and sewage. Johnson,[3] in a paper before the New England Waterworks Association, in 1905 first called attention to this, and cited certain experiments performed by him which apparently showed that B. coli could pass readily through parchment sacs, which retained perfectly their integrity as far as their dializing properties were concerned throughout the experiment. He showed that these organisms could be recovered from sterilized water in which parchment sacs filled with

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[1] *Jour. Infect. Dis.*, 1904, 1, p. 41.

[2] *Ibid.*, 1906, Supplement No. 2, p. 40.

[3] *Jour. N.E. Waterworks Assoc.*, 1905, 19, p. 506.

a starch solution and inoculated with B. coli had been immersed; also that B. coli appeared within sacs filled with water and sterilized, when such sacs were placed in sewage or polluted water. Johnson did not test the filtering properties of the sacs with B. typhosus, arguing that "if the less motile coli will pass through this membrane readily there is no room for reasonable doubt regarding the ability of the typhosus bacillus to act in a similar manner." This observer did not experiment with collodion membranes, however, and suggests that possibly this material "offers less opportunity for the exit of the highly motile typhoid bacilli through the walls of the sac than did the sacs of parchment."

More recently Todd[1] reports the result of some experiments on bacterial integrity of collodion and parchment membrane which seem to indicate that certain bacteria, notably those of intestinal origin, including the typhoid bacillus, dysentery, etc., pass readily through both collodion and parchment membranes of presumably perfect integrity. This observer tested "the growth of bacteria through collodion membranes" as follows: collodion sacs were made by Kellerman's[2] method and mounted on glass tubing. These were filled with broth and suspended in bottles containing broth. Air contamination was prevented by cotton packing and the complete outfit sterilized in the autoclave. Accidental contamination was guarded against by 24 hours' incubation in a thermostat. The sacs were then inoculated with the organism under observation and the outfit again placed in the thermostat. The passage of bacteria through the walls of the sac could be readily determined by the appearance of growth in the broth surrounding the sac. B. typhosus, B. dysenteriae, B. coli, B. aerogenes, B. cloacae, and organisms of similar character, also B. prodigiosus and B. pyocyaneus were recovered from the broth in which the inoculated sacs were immersed after a comparatively short incubation period. B. typhosus escaped from these sacs in 24 hours (average of eight tests), the shortest time being only four hours after inoculation. B. coli was recovered from the medium surrounding the sacs in 24 hours, B. prodigiosus in 24 hours (average of ten tests), and B.

[1] *Jour. Infect. Dis.*, 1909, 6, p. 368.

[2] *Jour. Applied Microscopy*, 1900, p. 2038.

pyocyaneus in 72 hours after inoculation. On the other hand, the non-motile cocci, B. subtilis, Bact. anthracis, and Sp. cholerae were retained in these sacs for several days. After the completion of the above experiments the sacs employed were retested by the air test, and if this was negative, by albumen solution.

"The direct passage of bacteria through collodion membranes" was tested by substituting distilled water for the broth used in the former experiment, thus removing conditions favoring growth. B. typhosus could be recovered from the water surrounding sacs inoculated with this organism in 14 hours, B. prodigiosus in 16 hours, and B. pyocyaneus in 27 hours. Todd also notes the passage of sewage bacteria through the walls of collodion sacs filled with sterile water and immersed in sewage, and in sterile water in which sacs filled with sewage had been placed. The earliest recovery of sewage organisms was made after 24 hours, and in no case did the membranes prevent the passage of these bacteria from the infected to the sterile liquid longer than 108 hours.

On the other hand, Frost,[1] in his studies on "The Antagonism of Certain Saprophytic Bacteria against B. typhosus," experienced little or no difficulty with collodion sacs which permitted the escape of organisms studied by him. The technic employed by him was very similar to that used by Todd. In many cases the medium in which the sacs were immersed was inoculated from one to six days before B. typhosus was introduced into the sacs themselves, so that the penetration of various saprophytic organisms into the sac could be readily detected. Sacs which became contaminated proved to be imperfect. Frost reports one experiment in which B. pyocyaneus was retained for a period of six months with a collodion sac, which was imbedded in gelatin instead of the usual broth. During this time the gelatin remained unchanged, altho luxuriant growth developed within the sac. After six months' growth the sac was intentionally ruptured and the surrounding gelatin rapidly liquefied by the escaping organisms.

Zeit[2] reported that collodion sacs immersed in river water became coated on the outside with a slimy deposit in about five days, but that the integrity of the sacs was not affected by it for several weeks.

[1] *Jour. Infect. Dis.*, 1904, 1, p. 599. [2] *Ibid.*, 1904, 1, p. 641.

In our hands the collodion sac method has given uniformly good results. Sacs filled with sterile water and immersed in water or sewage "showed no passage of bacteria through the sac membrane for a week or more."[1] While of course it is not possible to make perfect sacs at every trial, from 75 to 80 per cent of those made in this laboratory have proved entirely satisfactory. Furthermore, it is a simple matter to test the bacterial integrity of these containers by immersing them in polluted water or sewage for from 24 to 28 hours. Parchment membrane has not proved as satisfactory, for it is difficult to find sections of parchment tubing free from imperfection and of sufficient length to serve the purpose desired. For this reason parchment sacs were replaced by collodion containers in the latter series of experiments on the longevity of typhoid in water, in 1906.

With a view of proving that collodion sacs, similar to those employed in the typhoid studies referred to above, can be made so that they will remain unbroken for a period many times longer than that required to carry out the experiments cited, the following tests were made. Sacs were exposed to conditions most favorable to the passage of bacteria through the membrane, either by "growth" or "direct passage." The organisms tested were B. typhosus, B. coli, B. prodigiosus, and B. pyocyaneus and the bacteria of crude sewage and septic-tank effluent.

Sacs were made by the method recommended by Frost[2] in 1903, in test tubes 175 mm. in length and 25 mm. in diameter. The best results have been obtained from the use of a 10 per cent solution of Schering's collodion, in equal parts of absolute alcohol and ether. The sacs were air-dried for at least four or five hours before shrinking from the tubes with water. Air-drying for at least four hours is essential for the production of thin, tough membranes through which dialysis takes place most readily. Sacs dried for less than this time are frequently thicker and opaque, and dialysis takes place through them more slowly. After shrinking under water for several hours, the sacs could be slipped out of the tubes very easily, without sticking or tearing. The collodion tubes thus formed were trimmed to about five inches in length and slipped on to test tubes from which the bottoms had been cut, so that about three inches of the collodion sac protruded beyond the glass tubing. The sacs were tied firmly to the glass tubing with thread and the joints sealed tight with liquid collodion in order to exclude outside contamination. After filling the sacs with ordinary broth up to the supporting tube, they were plugged with cotton and suspended in Phillips beakers of 250 c.c. capacity, which were also filled with broth. These tubes were held firmly in

[1] *Jour. Infect. Dis.*, 1906, Supplement No. 2, p. 40.

[2] *Amer. Pub. Health Assoc. Papers and Reports*, 1903, 28, p. 536.

place by a tight packing of cotton around them in the neck of the beaker. All the dialyzing membrane was immersed in the culture medium which was protected from air infection by the cotton in the neck of the flask (Fig. 1, Sac B). The whole outfit was sterilized in an autoclave for 20 minutes at 15 pounds pressure. The sterilized containers and sacs were allowed to stand at room temperature for four or five days in order to be certain that no accidental contamination had taken place. The sacs were then inoculated with the organism to be studied. The inoculated sacs were kept at room temperature and daily observations were made. As long as the medium in which the infected sacs were immersed remained clear and showed no other evidence of growth, it was taken for granted that the bacterial integrity of the sacs had been maintained. When growth developed in the broth surrounding the sacs, tests were made to determine the character of the organisms causing the change in the medium. If the sacs retained their integrity for 60 days after inoculation, most of the experiments were discontinued and plate cultures made to recover the original organisms from the inoculated sacs and to prove the absolute sterility of the medium in which they had been immersed. A few experiments were allowed to run on indefinitely, and, at the present writing, June 20, there remain 12 sacs which have maintained their integrity for nearly six months. The results of these experiments are arranged in the following table:

TABLE I.

SHOWING LENGTH OF TIME COLLODION SACS RETAIN THEIR BACTERIAL INTEGRITY AFTER INOCULATION WITH VARIOUS ORGANISMS.

No. of Sac	Organism Tested	Date of Inoculation	Organism First Isolated from Medium Surrounding Sac
1	B. pyocyaneus	December 8	December 14
2	"	" "	" 16
3	"	" "	Not in 60 days
4	"	" 21	" " " " *
5	"	" "	" " " "
6	"	" "	" " " "
7	B. prodigiosus	" 15	January 25
8	"	" "	Not in 60 days *
9	"	" "	" " " "
10	"	" "	" " " "
11	B. coli	" "	January 25 *
12	"	" "	Not in 60 days *
13	"	" "	" " " "
14	"	" "	" " " "
15	"	" "	" " " "
16	B. typhosus	" 18	January 31
17	"	" "	February 10
18	"	" "	Not in 60 days
19	"	" "	February 10
20	"	" "	Not in 60 days *
21	"	" "	" " " " *
22	"	" "	" " " " *
23	"	January 10	" " " " *
24	"	" "	" " " " *
25	"	" "	" " " " *
26	"	" 31	" " " " *
27	"	" "	" " " " *
28	"	" "	" " " "
29	"	" "	" " " "
30	"	" "	" " " "
31	Crude sewage and typhosus	" "	" " " "
32	" " " "	" "	" " " "
33	" " " "	" "	" " " " *
34	Septic-tank effluent and typhosus	" "	" " " "
35	" " " " "	" "	" " " "

* Sacs marked with a star remain intact at date of writing, June 20.

From the results above it will be seen that the majority of collodion sacs tested retained their bacterial integrity for 60 days or longer. Six sacs were inoculated with B. pyocyaneus. Growth in the medium surrounding one of these developed in six days; the organism escaped from another in eight days. B. pyocyaneus was recovered in pure culture from the test broth in each case. The six-day sac had not been tightly sealed to the supporting tube and had slipped down into the broth surrounding it, so that the organisms escaped from the top of the sac rather than through the walls. This was proved to be undoubtedly the case, for after washing with distilled water this sac was sealed on to its glass support with fresh collodion and sterilized as before. The sac was reinoculated with B. typhosus and retained the organism for 60 days, when the experiment was discontinued. The eight-day sac proved to be defective. The four remaining sacs inoculated with B. pyocyaneus held perfectly for 60 days; three of them were discontinued but the fourth was allowed to run on and held germ-tight for 127 days. Subcultures from this sac showed actively motile bacilli, developing the deep-green pigment characteristic of B. pyocyaneus. The pigment of this organism diffuses readily through the walls of collodion sac and can be recognized in broth surrounding it within 36 hours after inoculation of the sac. After a month or more the medium becomes a very deep green but perfectly clear if the sac holds. The broth, in which the sac kept for 171 days was immersed, assumed a black color and was nearly opaque (Fig. 1, Sac I).

B. prodigiosus is also retained by collodion sacs for a considerable time. Of the four tests made, one sac held for 41 days and three over 60. Up to the time of writing one sac has held 174 days. Pure cultures of B. prodigiosus were obtained from this sac 170 days after inoculation (Fig. 1, Sac H).

Four out of five sacs, inoculated with different strains of B. coli, held for 60 days, and two sacs which were kept under observation longer retained the organism for 174 days. Pure cultures of B. coli were recovered from both these sacs (Fig. 2, Sac G).

Fifteen sacs were inoculated with B. typhosus in pure culture. Of these one held 49 days, two 59 days, and twelve over 60 days.

Eight of these sacs were kept under observation and still retain the organism with which they were inoculated at time writing (137–270 days) (Fig. 2, Sac F, Fig. 3, Sacs D, E).

In addition to the pure cultures used in these experiments, five sacs were filled with sewage and heavily seeded with a suspension

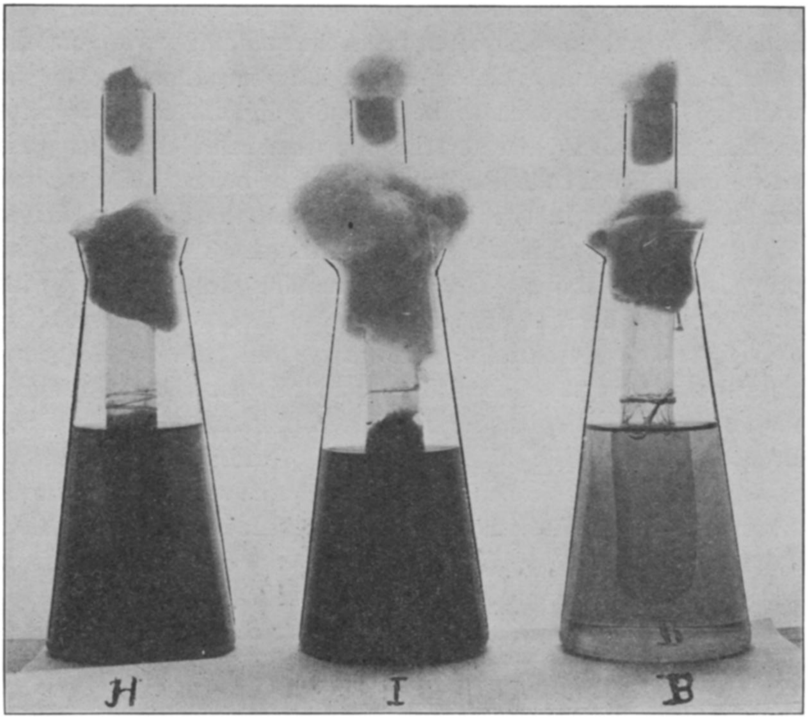

FIG. 1.—Photographs of sacs inoculated with B. prodigiosus and B. pyocyaneus. Photographs taken 71 and 65 days, respectively, after inoculation.

Sac H. Inoculated with B. prodigiosus December 15, 1909. The medium surrounding this sac assumed a red-brown color but is perfectly clear and the outline of the sac can be faintly seen within it.

Sac I. Inoculated with B. pyocyaneus December 21, 1909. The broth in which this sac was immersed became almost opaque and black in color from the diffusion of soluble pigments through the walls of the sac. The broth, however, remained perfectly sterile.

Sacs H and I. Retained their bacterial integrity at the time of writing, June 20.

Sac B. Control. Uninoculated sac in sterile broth.

of typhoid organisms. (After sterilization, the broth filling the sacs was carefully removed with a large sterile pipette and replaced with sewage.) Three sacs were filled with crude sewage, and two

with septic-tank effluent. All five sacs held 60 days and one (septic-tank effluent plus typhoid) retains its bacterial integrity at time of writing, 140 days after inoculation (Fig. 4, Sacs A, C).

In addition to the above experiments, two sacs were made in Erlenmeyer flasks of 400 and 250 c.c. capacity, respectively. The

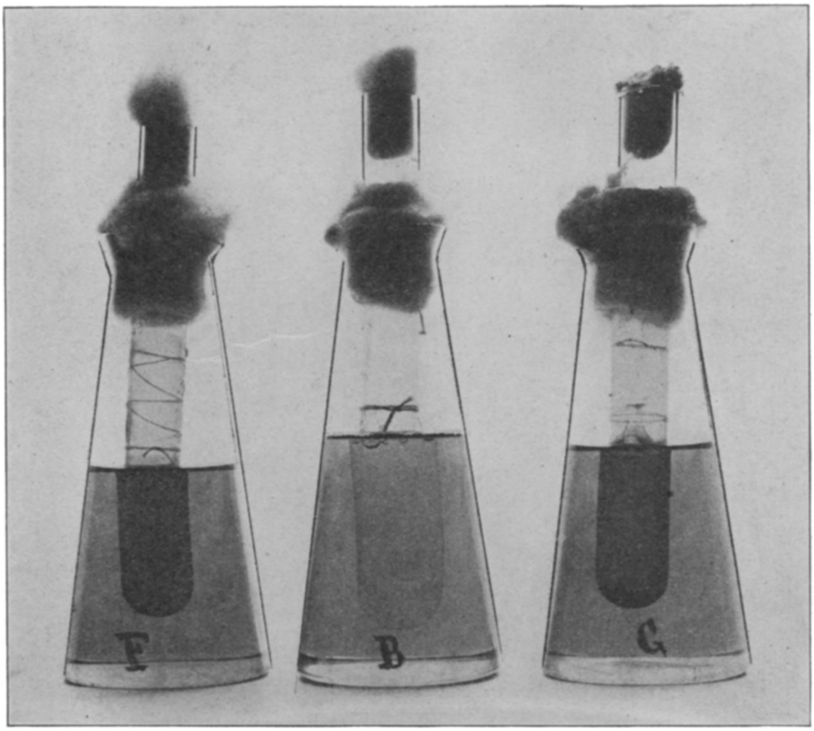

FIG. 2.—Photographs of sacs inoculated with B. typhosus and B. coli. Photographs taken 68 and 71 days, respectively, after inoculation.

Sac F. Inoculated with B. typhosus December 18, 1909.

Sac G. Inoculated with B. coli December 15, 1909.

The sacs showed very heavy growth and deposit at the bottom of the tubes, outlining the sacs sharply in the clear broth in which they were immersed. The beakers in which these sacs were suspended were sealed with paraffin, thus they do not show the evaporation noticed in Fig. 3, Sacs D and E. These sacs retained their bacterial integrity June 20.

Sac B. Control. Uninoculated sac in sterile broth.

400 c.c. collodion flask was sealed on to a glass tubing of slightly smaller diameter than the neck of the flask. This large sac was strong enough to support without rupture the weight of broth neces-

sary to fill it to the neck. This sac was immersed in broth in a 1,500 c.c. beaker, and held firmly in position by strong wire supports. A layer of cotton between two pieces of gauze covered the mouth of the beaker and protected the medium surrounding the sac from air contamination. The glass tube, supporting the sac, passed

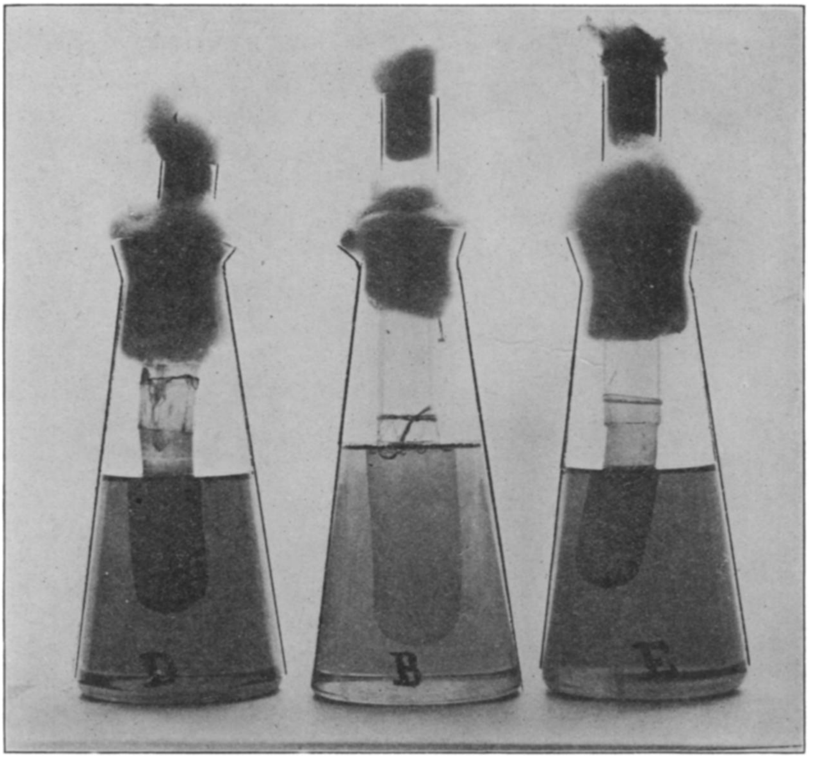

FIG. 3.—Photographs of sacs inoculated with B. typhosus. Photographs taken 68 days after inoculation.

Sacs D and E inoculated with B. typhosus December 18, 1909. Note the accumulation of growth in the bottoms of these sacs; also the dry shrunken upper portion due to evaporation of the medium in the flask. These sacs are still germ-tight June 20.

Sac B. Control. Uninoculated sac in sterile broth.

through a hole in the center of the gauze and, after sterilization, a tight joint was made by sealing with paraffin. The whole outfit was autoclaved for 45 minutes at 15 pounds pressure. After sterilization the outfit was allowed to stand at room temperature for a week in order to insure against accidental contamination. The

sac was then inoculated with B. prodigiosus, maintaining its bacterial integrity for 61 days. During this time so much of the broth evaporated from the beaker that the sac finally ruptured, presumably from the weight of liquid and accumulated mass of organisms which filled the bottom of the sac to a depth of about

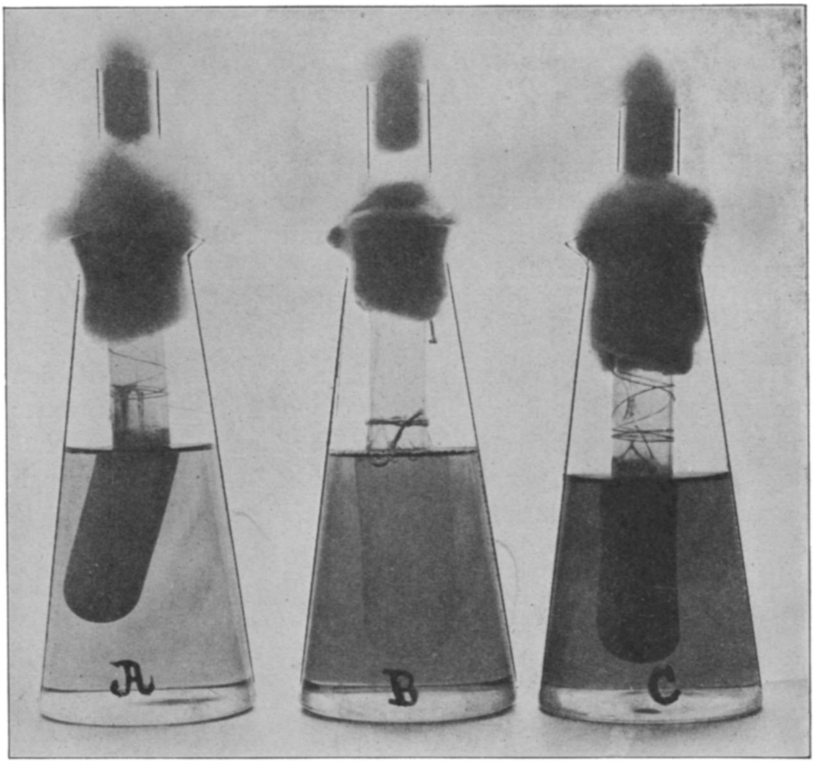

FIG. 4.—Photographs of sacs filled with crude sewage and septic-tank effluent and inoculated with B. typhosus. Photographs taken 24 days after inoculation.

Sac A. Filled with septic-tank effluent and inoculated with B. typhosus January 31, 1910. The sac became opaque on account of the very luxuriant growth which clung to the sides of it and is sharply defined in the sterile broth in which it is immersed. This sac is still intact June 20.

Sac C. Filled with crude sewage and inoculated with B. typhosus January 31, 1910. This sac also contains a very heavy growth.

Sac B. Control. Uninoculated sac in sterile broth.

half an inch. The sac formed in the 250 c.c. flask was mounted in a manner similar to the first. This sac was inoculated with B. typhosus and retained its integrity for 48 days.

The cultures of B. typhosus used in these experiments were sub-

cultures from a strain obtained from Parke, Davis & Co., and are used by the Wisconsin State Hygienic Laboratory for Widal determinations. The colon cultures were young and vigorous strains freshly isolated from feces.

CONCLUSIONS.

The results obtained from these experiments are entirely at variance with the results reported by Todd in 1909, and correspond perfectly with those obtained by us in former tests of the bacterial integrity of collodion sacs.

By Frost's method it is possible to make collodion sacs which will retain their bacterial integrity for several months. B. typhosus, B. coli, B. prodigiosus, B. pyocyaneus, and the bacteria of crude sewage or septic-tank effluent will not escape from sacs made by this method, either by "growth" or "direct passage through the walls."

The reliability of the results obtained by the use of collodion sacs for the determination of the longevity of B. typhosus in water and sewage cannot be seriously questioned on the ground that they do not take into account the escape of B. typhosus through the walls of the sacs during the coarse of these experiments.